TENT COOPERATION TREATY

REC'D 12 MAR 2001 WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 91874	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/AU99/00995**	International Filing Date *(day/month/year)* 11 November 1999	Priority Date *(day/month/year)* 11 November 1998
International Patent Classification (IPC) or national classification and IPC **Int. Cl.** [7] G01N 33/10, 33/573, 33/577, C07K 14/40		
Applicant QUALITY WHEAT CRC LIMITED et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

Date of submission of the demand 26 May 2000	Date of completion of the report 26 February 2001
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **ROSS OSBORNE** Telephone No. (02) 6283 2404

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , received on with the letter of

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages
[ ] the claims, Nos.
[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims **1-22**	**YES**
	Claims	**NO**
Inventive step (IS)	Claims	**YES**
	Claims **1-22**	**NO**
Industrial applicability (IA)	Claims **1-22**	**YES**
	Claims	**NO**

2. Citations and explanations (Rule 70.7)

NOVELTY(N) Claims 1-22

None of the citations teach a method of detecting pre-harvest sprouting using a monoclonal to alpha amylase or teach antibodies to the specific epitopes defined by the claims.

INVENTIVE STEP (IS) Claims 1-22

D1. Lecommandeur et al, Hybridoma (1990)

D2. Sander et al, J. Immunol. Methods (1997)

D3. AU 93559/1998

It is considered that the skilled worker in the art developing a assay for pre-harvest sprouting would as a matter of routine development produce the claimed assay or one based on other alpha amylase epitopes that were functionally equivalent.

The skilled addressee faced with the problem of detecting pre-harvest sprouting would be aware of that alpha amylase is a marker (as mentioned in both D1 and D3).

In searching for solutions to developing an assay for alpha amylase the skilled addressee would find documents D1 and D2. The development of an ELISA assay using monoclonals to alpha amylase to (broadly speaking) assess cereal quality is taught in D1 and the benefits of a two site assay for assaying the related Aspergillus amylase is taught in D2.

On this basis the person skilled in the art would be led to develop a two site ELISA assay to detect pre-harvest sprouting with the epitopes chosen to have the appropriate level of selectivity.

The monoclonals of the present invention were prepared using as a mix of antigens both high and low pI amylases, a procedure that would appear a totally conventional approach. A panel of antibody producing hybridomas were obtained and the vast majority were unsuitable, again an expected result, but as enough hybridomas were screened at least one was found suitable. No problems in the development of the assay have been cited which appear to have required inventive ~~merit~~ to overcome. Of course it could not be predicted which specific epitopes would be suitable, but no surprising advantages of these epitopes are cited, rather it appears they are the first the applicant found that were suitable for a commercial form of this assay. The particular epitopes against which the monoclonals are claimed appear to be simply the sites of binding with the first suitable antibodies identified in this successful but routine development of an assay.

For these reasons the claims lack inventive step

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 June 2000 (14.06.00)	
International application No. PCT/AU99/00995	**Applicant's or agent's file reference** 91874
International filing date (day/month/year) 11 November 1999 (11.11.99)	**Priority date** (day/month/year) 11 November 1998 (11.11.98)
Applicant SKERRITT, John, Howard	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

26 May 2000 (26.05.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer Nestor Santesso Telephone No.: (41-22) 338.83.38

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/10, 33/573, 33/577, C07K 14/40 | A1

(11) International Publication Number: WO 00/28319

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/AU99/00995

(22) International Filing Date: 11 November 1999 (11.11.99)

(30) Priority Data:
PP 7058 11 November 1998 (11.11.98) AU

(71) Applicant (*for all designated States except US*): QUALITY WHEAT CRC LIMITED [AU/AU]; Riverside Corporate Park, Gate 1, 51 Delhi Road, North Ryde, NSW 2113 (AU).

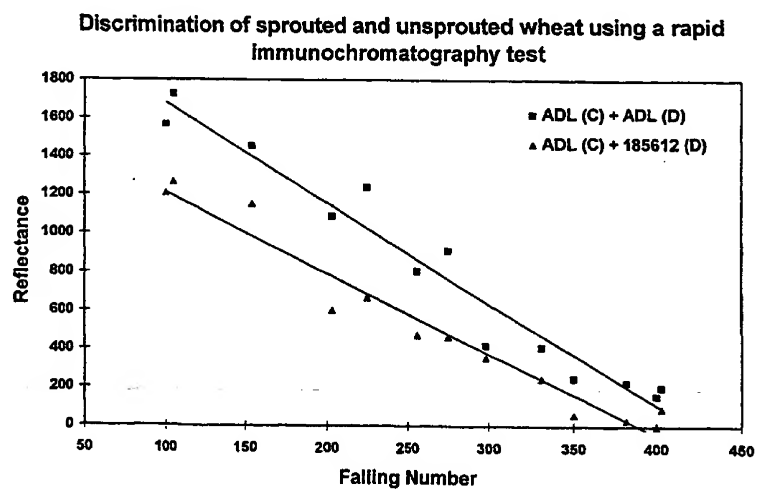
(72) Inventor; and
(75) Inventor/Applicant (*for US only*): SKERRITT, John, Howard [AU/AU]; 38 Booth Crescent, Cook, ACT 2614 (AU).

(74) Agent: F B RICE & CO.; 605 Darling Street, Balmain, NSW 2041 (AU).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: DETECTION OF PREHARVEST SPROUTING IN CEREAL GRAINS



(57) Abstract

A two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising: (i) exposing said test sample to a first ("capture") antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present, (ii) subsequently exposing bound alpha-amylase, if any, to a second ("detection") antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and (iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences: IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80 %, more preferably ≥ 90 %, sequence identity. The immunoassay is useful for detecting weather damage (i.e. preharvest sprouting) in cereal grain.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

DETECTION OF PREHARVEST SPROUTING IN CEREAL GRAINS

Field of the Invention:

This invention relates to a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase. The invention allows for the identification of "weather damage" in cereals (especially wheat).

Background of the Invention:

Weather damage or preharvest sprouting in wheat, is caused by the action of hydrolytic enzymes (amylases, proteases and lipases) in the grain endosperm. These enzymes (triggered by rain at or just before harvest), accelerate the breakdown of starch granules and protein in the endosperm of germinating grain (Meredith, P.; Pomeranz, Y. Advances in Cereal Science and Technology 7 (1985) 239-299). Wheat that is weather-damaged has a significantly lower market value as a result of being rendered unsuitable for human consumption. This is because the products that are made from sprouted wheat, for example breads, have grey colour, crumb texture, loaf structure and volume or in the case of noodles, poor colour and cooking qualities, due to the action of these hydrolytic enzymes, which include alpha-amylases (Orth, R.A.; Moss, H.J. Proceedings of the Fourth International Conference on Pre-harvest Sprouting, D. Mares (Ed.) Westview Press, Boulder, CO., USA (1987) 167-175; Derera, N.F. (Ed.) Preharvest sprouting in cereals, CRC Press Inc., Boca Raton, FL, USA (1989)).

At grain delivery to the silo or elevator or during harvesting, mixing of a small quantity of damaged grain with larger amounts of sound grain can lead to downgrading of all of the grain. The necessity for accurately discriminating sprouted from sound wheat highlights the

need for a quick, easy and reliable test for preharvest sprouting. The most common method for detecting preharvest sprouting at elevators involves the measurement of alpha-amylase activity using the "Falling Number" method, in which the consequences of enzymic hydrolysis of starch caused by amylase production is assessed as the time required for a plunger to fall through a heated slurry of wholemeal and water (Hagberg, S. Cereal Chemistry 37 (1960) 218; Perten, H. Cereal Chemistry 41 (1964) 127-140). However, the capital cost of the instrument means that it is only feasible to install them at a limited number of mills or major grain silos or elevators. The method is also relatively low in throughput and results can be affected by variation in starch pasting characteristics (D'Appolonia, B.L.; Macarthur, L.A., Pisesookbuntererng, W.; Ciacco, C.F. Cereal Chemistry 59 (1982) 254-257; Ringlund, K. Proceedings of the Third International Symposium on Pre-Harvest Sprouting in Cereals, J.E. Kruger and D.E. LaBerge (Eds.) Westview Press, Boulder, CO, USA, (1983) 111-118).

The cheaper option of visual assessment is both unreliable and not objective (Jensen, S.A.; Munck, L.; Kruger, J.E. Journal of Cereal Science 2(1984) 187-201), while other methods such as the Rapid ViscoAnalyzer (Ross, A.S.; Orth, R.A.; Wrigley, C.W. Proceedings of the Fourth International Symposium on Pre-Harvest Sprouting in Cereals, D.J. Mares (Ed.) Westview Press, Boulder, CO, USA (1987) 577-583) and Near Infrared analysis (Czuchajowska, Z.; Pomeranz, Y. Preharvest Sprouting in Cereals 1992, M.K. Walker-Simmons and J.L. Ried (Eds.) American Association of Cereal Chemists, St Paul, MN, USA (1992) 409-416), although faster, involve high capital cost. Near Infrared predictions of Falling Number are also of relatively low precision and can only discriminate

relatively large differences in Falling Number (Osborne, B.G. Journal of the Science of Food and Agriculture, 35 (1984) 106-110; Czuchajowska, Z.; Pomeranz, Y.; Preharvest Sprouting in Cereals 1992, M.K. Walker-Simmons and J.L. Ried (Eds.) American Association of Cereal Chemists, St Paul, MN, USA (1992) 409-416; Shashikumar, K.; Hazleton, J.L.; Ryu, G.H.; Walker, C.E. Cereal Foods World 38 (1993) 264-269). Direct enzyme activity assays for alpha-amylase (Barnes, W.C.; Blakeney, A.B. Die Starke 6 (1974) 193-197; McCleary, B.V.; Sheehan, H. Journal of Cereal Science 6 (1987) 237-251) are not suited for silo (elevator) or on-farm use due to a need for technical expertise and equipment such as waterbaths and filtration devices.

Immunoassays provide alternative methods for detection of preharvest sprouting through the use of antibodies that are specific for alpha-amylase isozymes. Alpha-amylases are considered to be the most appropriate targets for a test because: 1. they are relatively abundant, 2. they are synthesized early in the preharvest sprouting sequence (Corder, A.M., and Henry, R.J. Cereal Chemistry 66 (1989) 435-439), 3. they are responsible for many of the quality defects that occur when end products are prepared from sprouted wheat, and 4. the basis of the measurements in the "industry standard test" (Falling Number) is changes in the viscosity of a wholemeal-water slurry due to the presence of carbohydrate-degrading enzymes such as amylases. Earlier research has shown that specific antisera can be developed for separate recognition of the two major groups of alpha-amylase isozymes (Daussant, J.; Renard; H.A. Cereal Researh Communications 4 (1976) 201-212; Lazarus, C.M.; Baulcombe, D.C.; Martienssen, R.A. Plant Molecular Biology 5 (1985) 13-24). Immunoassay techniques have an added potential

advantage over enzyme activity assays, in that by using appropriate amylase antibodies it should be possible to specifically measure different amylase isozyme families. Immunoassay kits are generally quite robust and suitable for shipping and use in harsh environments, and can be used by individuals with little training. Such tests could not only be used by grain handlers or traders at silo (elevator) delivery of grain, but also by individual wheatgrowers. This would allow them to detect sprouting on-farm prior to harvesting in order to prevent contamination of sound wheat by sprouted grain.

The most sensitive, specific and quantitative immunoassays require the use of both a solid-phase bound antibody and a labelled detection antibody in a "two-site" assay. The detection antibody may be labelled with an enzyme, coloured particle or sol, or radioactive element or fluorophore. However, for field use without special equipment, the most useful methods are those in which the test result can be interpreted visually. The present invention relates to the development of two-site immunoassays for the qualitative or quantitative detection of alpha-amylase.

Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising;

(i) exposing said test sample to a first ("capture") antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present,

(ii) subsequently exposing bound alpha-amylase, if any, to a second ("detection") antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and

(iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80%, more preferably ≥ 90%, sequence identity.

Preferred variant sequences include those that differ from the amino acid sequences IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3) in one or more conservative amino acid substitution(s). The conservative amino acid substitutions envisaged are:- G,A,V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,Nα-alkalamino acids.

Preferably, either of said first or second epitopes is a conformational epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

More preferably, either of said first or second epitopes is a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

The two-site immunoassay of the present invention may be performed in accordance with any of the formats well known in the art. Particularly preferred formats include

the sandwich enzyme-linked immunosorbent assay (ELISA) and immunochromatography (IC).

In the ELISA format, the first antibody or fragment thereof is provided bound to a solid support such as a microwell plate, membrane, beads, particles or suitable sensor. It is preferable to use a blocking agent to prevent non-specific binding and to conduct the ELISA assay with washing steps as is well known in the art.

In the IC format, the second antibody or fragment thereof is provided bound to a solid support such as porous test strip. As is well known in the art, it is possible to conduct IC assays without the use of a blocking agent or washing steps.

At least one of the first and second antibodies or fragments thereof is/are preferably selected from monoclonal antibodies or fragments thereof (e.g. Fab and $F(ab')_2$), recombinant antibodies or fragments thereof and recombinant antibody fragments (e.g. scFv). These provide significant commercial advantages over, for example, polyclonal antibodies. First, they recognise a limited number of epitopes and, for that reason, do not form aggregating complexes which can compromise ELISA or IC performance. Secondly, they are constant and reproducible reagents.

Detection of binding of the second antibody or fragment thereof may be achieved through the use of a readily detectable label such as a detectable enzyme (e.g. horseradish peroxidase or alkaline phosphatase), radioisotope (e.g. P^{32} or S^{35}) or luminescent or fluorescent label. Detection of binding of the second antibody might also be achieved through other means such as immunochromatography and agglutination.

The two-site immunoassay of the present invention is particularly suitable for the qualitative or quantitative

detection of alpha-amylase in a cereal grain (e.g. wheat including bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum* var. durum) and club wheat (*Triticum compactus*); rye (*Secale cereale*); triticale (*Triticosecale* species); barley (*Hordeum vulgare*) and related cereals (i.e. members of the *Triticeae* family), and thereby provides for the qualitative or quantitative detection of weather damage.

The test sample utilised in a two-site immunoassay for this purpose is preferably an aqueous extract from grain or, more preferably, grain meal or flour. As is described in greater detail below, alpha-amylase may be readily extracted from grain meal or flour with a dilute solution of NaCl or $CaCl_2$.

When used for the quantitative detection of alpha-amylase in cereal grain, the two-site immunoassay further comprises a step of comparing the level of detected binding of the second antibody or fragment thereof against a suitable standard. Preferably, the level of detected binding of the second antibody or fragment thereof is positively correlated with alpha-amylase enzyme activity and negatively correlated with Falling Number.

In a second aspect, the present invention provides a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80%, more preferably ≥ 90%, sequence identity.

Preferred variant sequences include those that differ from the amino acid sequences IDRLVSIRTRGQIHS (SEQ ID NO:

1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3) in one or more conservative amino acid substitution(s). The conservative amino acid substitutions envisaged are:- G,A,V,I,L,M; D,E; N,Q; S,T; K,R,H; F,Y,W,H; and P,Nα-alkalamino acids.

Preferably, the monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner of the present invention specifically or preferentially binds to a conformational epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

More preferably, the monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner of the presentinvention specifically or preferentially binds to a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2) and VNWVNKVGGS (SEQ ID NO: 3).

In a third aspect, the present invention provides a kit for performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to the second aspect.

For performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a cereal grain, the kit may further comprise a container including an aqueous extraction agent for extracting alpha-amylase from grain, grain meal or flour.

Throughout this specification, unless the context requires otherwise, the term "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

The term "specifically binds" as used herein is intended to refer to binding characteristics of antibodies and fragments thereof which bind exclusively to the defined epitope on alpha-amylase or with only negligible cross-reaction with epitopes on other cereal grain substituents.

The term "preferentially binds" as used herein is intended to refer to binding characteristics of antibodies and fragments thereof which bind strongly to the defined epitope on alpha-amylase and to a lesser extent with epitopes on other cereal grain substituents.

The term "recombinant antibody", refers to an antibody that has been expressed from a host cell culture that has been transformed with an isolated, manipulated or synthesised expressible gene encoding the antibody. Methods for producing such recombinant antibodies are described in Pluckthun, A. Bio/Technology 9, 545-551 (1991).

The term "recombinant antibody fragment", refers to an antibody fragment that has been expressed from a host cell culture that has been transformed with an isolated or synthesised expressible gene encoding the antibody fragment. Examples of such recombinant antibody fragments are single chain Fv (scFv) antibody fragments. Methods for producing scFvs are described in Pluckthun, A. Bio/Technology 9, 545-551 (1991) and US Patent No. 4,946,778.

The invention is hereinafter described by reference to the accompanying figures and the following non-limiting examples describing the preparation and characterisation of suitable antibodies, their utilisation in immunoassays, and the use of immunoassays to quantify differences in alpha-amylase in wheat samples.

Brief description of the accompanying figures:

Figure 1 shows the titration of monoclonal and polyclonal antibodies to alpha-amylase using indirect ELISA. ELISA plates were coated with 1 µg purified amylase per microwell (high plus low pI isozymes from cv. Janz). Data are the mean ± SD of 3 replicates.

Figure 2 shows the performance of antibody combinations in microwell two-site ELISAs with extracts of an unsprouted (Falling Number 403) and moderately sprouted (Falling Number 187) sample. Data are shown for three capture antibodies (185612, ADL, ALI) and eight detection antibodies.

Figure 3 shows amino acid sequences with high-pI alpha-amylase clone Amy-1 13/1 recognised by seven antibodies.

Figure 4 shows the effect of antigen partial denaturation with urea on detection of high-pI alpha-amylase by three antibodies.

Figure 5 shows the discrimination of sprouted and unsprouted wheat through relationships between ELISA absorbance using a rapid tube and Falling Number for wheat samples from elevators in Queensland, with A. 59 grain samples and an assay using immobilised ALI antibody and enzyme-labelled 185612, and B. 130 grain samples and an assay using immobilised ALI antibody and enzyme-labelled ALI antibody. Samples were analyzed in duplicate in two

separate runs and raw absorbances standardized to the absorbance of a standard of Falling Number 187 units included in each run.

Figure 6 shows the relationship between immunochromatography band intensity (reflectance) and Falling Number for 13 wheat samples using antibody ADL as the capture (C) antibody plus 185612 or ADL as gold-labelled detection (D) antibody.

Figure 7 shows the within-day precision of immunochromatography tests (mean ± standard deviations of results of 5 tests) using different antibody combinations and moderately sprouted (Falling Number 154 seconds), mildly sprouted (Falling Number 275 seconds) and unsprouted (Falling Number 382 seconds) wheat grain.

Examples:

Example 1: Production of antibodies to alpha-amylases and analysis of their specificities

Amylase purification

Grain from two wheat cultivars (Janz and Osprey) was germinated for 4 days at 20 °C. Germinated grain was frozen by immersion in liquid nitrogen and freeze-dried. Roots and shoots were removed and the grain was ground into wholemeal flour using a Jupiter Electric Cereal Grinder (JUPITER, Schorndorf, Germany). Alpha-amylase was extracted by stirring wholemeal flour in 20 mM sodium acetate buffer, pH 5.5 containing 1 mM $CaCl_2$ (extraction buffer) at 5 °C for 1 hour. All subsequent steps were performed at 5 °C. The extract was centrifuged (48,000 *g*, 30 min), and the resulting supernatant subjected to a 20 - 60 % ammonium sulfate precipitation. The material that precipitated between these salt concentrations was

redissolved and dialyzed against the extraction buffer. The dialyzed extract was filtered using a 0.8 μm filter and purified using affinity chromatography.

A mixture of both high and low pI isozymes of alpha-amylase was isolated by β-cyclodextrin affinity chromatography. The affinity column (12 x 3 cm) consisted of epoxy-activated Sepharose 6B coupled to cyclohepta-amylose (β-cyclodextrin); Sepharose 6B was activated using 1,4-butanedioldiglycidyl ether (Sigma, St Louis, MO) according to the methods of Sundberg, L.; Porath, J. Journal of Chromatography 90 (1974) 87-98) and coupled to cycloheptaamylose using the method of Vretblad, P. FEBS Letters 47 (1974) 86-89. Alpha-amylase was eluted with 20 mM sodium acetate buffer (pH 5.5), containing 1 mM $CaCl_2$, 0.5 M NaCl and 8 mg/mL of cycloheptaamylose, and dialysed against 10 mM sodium acetate buffer (pH 5.5), containing 1 mM $CaCl_2$ and reduced in volume by ultrafiltration.

Separation of high and low pI isozymes of alpha-amylase was achieved using one of three methods, either 1) ion-exchange chromatography (Lecommandeur, D. Journal of Chromatography 441 (1988) 436) using CM-Sepharose CL-6B, equilibrated with 20 mM acetate buffer, pH 4.8, containing 1 mM calcium chloride and eluted with a 0 - 0.3 M NaCl gradient, 2) preparative isoelectric focussing using a Rotofor device (Hochstrasser A.C. et al; Applied and Theoretical Electrophoresis, 1 (1991) 333-337) and pH 3-10 carrier ampholytes (Biorad, Hercules, CA, USA), or 3) immunoaffinity chromatography using a column prepared by coupling a monoclonal antibody prepared to the high pI alpha-amylase isozyme of barley (Gibson, C.E., Evans, D.E., MacLeod, L.C., Symons, M.H., Marschke, R.J., Jarratt, S., Dalton, M.R., Lance, R.C.M., Skerritt, J.H., Henry, R.J. and Fincher, G.B. Proceedings of the 44th

Australian Cereal Chemistry Conference, Royal Australian Chemical Institute, Melbourne, 44 (1994) 174-179), to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). A crude extract of amylase purified using the cycloheptaamylose method, was dialyzed against the immunoaffinity column equilibration buffer (50 mM Tris, pH 7.6 containing 5 mM $CaCl_2$ and 0.5 M NaCl). High pI alpha-amylase was eluted with 2.5 M KSCN containing 5 mM $CaCl_2$ and 0.5 M NaCl. The unbound fraction (thought to contain low pI amylase) was collected during loading onto the column. This fraction along with the eluted enzyme was dialyzed and concentrated as described above.

The purity of alpha-amylase prepared from crude wheat extract by affinity chromatography was shown by SDS-PAGE to contain a single band with with an approximate molecular weight of 44,000, in agreement with previous reports (Hill, R.D.; MacGregor, A.W. Advances in Cereal Science and Technology 9 (1988) pp 217-261. Isoelectric focusing of purified alpha-amylase, revealed the presence of approximately 6 to 8 bands between pH 6-7 (high pI isozymes, products of *alpha-amy 1* genes on group 6 chromosomes) and approximately four to six less strongly stained bands between pH 4.5 - 5.5 (low pI isozymes; products of *alpha-amy 2* genes on group 7 chromosomes). Two hundred grams of germinated grain yielded approximately 24 mg of a mixture of high and low pI amylase and approximately 14 mg of high pI amylase.

Production of Monoclonal and Polyclonal Antibodies to Alpha-Amylase

Polyclonal antisera were produced to the high pI amylase isozymes and to a mixture of both the high and low pI isozymes of the enzyme. Rabbits were given an initial injection of 1 mg protein (mixed 1:1 in Freund's Complete Adjuvant, FCA; Sigma) split between three injection sites

in the muscles of both hind legs and subcutaneously in the neck area. Rabbits were boosted 14 and 28 days after the initial injection (500 µg protein in Freund's Incomplete Adjuvant, FICA), and blood was collected 10 days after the third booster. Sera were tested for antibodies to alpha-amylase using indirect ELISA (see below), and rabbits were boosted thereafter at monthly intervals with 500 µg protein in FICA. Antibodies were purified from rabbit sera using Gamma Bind IgG affinity chromatography.

Monoclonal antibodies (MAb) to-alpha amylase were produced by immunizing BALB/c mice with a mixture of both high and low pI isozymes of the enzyme (purified separately from both Janz and Osprey cultivars). Mice were given an initial intraperitoneal (IP) injection of 200 µg total protein mixed 1:1 in FCA. This was followed by two IP injections (in FICA) of 100 µg protein at 2-week intervals. At 4 weeks after the third injection, mice were given a final IP booster of 200 µg amylase per mouse 3 days prior to fusion. The quantity of enzyme used for immunisation was optimised in initial experiments in which 3 groups of mice (2 mice/group) were immunised initially with either 200, 40 or 10 µg total protein, and subsequently with either 100, 20 or 5 µg total protein. Blood was collected from all mice by tail bleed at 10 days after the third injection and tested for antibodies against alpha-amylase by indirect ELISA. Hybridoma production was carried out according to the general methods of Skerritt, J.H.; and Underwood, P.A. Biochimica et Biophysica Acta 874 (1986) 245-254, and supernatants secreted by the resulting hybridoma cells were tested for specificity to alpha-amylase using indirect ELISA (see below). Positive cell lines were subcloned cloned and

expanded by ascites, and antibodies isotyped using a Mouse-Typer Sub-isotyping Kit (BioRad, Hercules, CA). Several high-titre independent cell lines which secreted high-titer antibodies were isolated: 15764, 15689, 10185 and 10413 (all IgM,κ) and 15724 and 185612 were IgG_1, κ.

Monoclonal and polyclonal antibodies were tested for specificity for alpha-amylase using semi-dry immunoblotting of SDS-PAGE gels and passive immunoblotting of isoelectric focussing (IEF) gels. SDS-PAGE gels (12 % T, 2 % C gel, run 1500 Vhr) were loaded with a crude extract of alpha amylase and electrophoresis and immunoblotting (for 4 h at 250 mA/gel) onto nitrocellulose was carried out according to Andrews, J.L.; and Skerritt, J.H. Journal of Cereal Science, 23 (1996) 61-72. IEF used 7.5% T, 3% C polyacrylamide gels (0.5 mm) with an ampholyte pH gradient of 3-10. 1 M sodium hydroxide and 1 M phosphoric acid were used as cathode and anode solutions respectively, and gels were run at 4 °C under constant power (8-10 W) for 3 hours after loading with purified ∝-amylase. Membranes from both procedures were blocked with 3 % (w/v) bovine serum albumin (BSA) in 50 mM sodium phosphate-buffered saline, pH 7.2 (PBS), probed with MAbs or PAbs, and detected with alkaline phosphatase-labeled second antibodies from Promega (Madison, WI, USA).

On immunoblots of SDS-PAGE gels, both the polyclonal antiserum to high/low pI amylase and to high pI amylase primarily detected the M_r 44,000 polypeptides corresonding to alpha-amylase. Weak reaction with other polypeptides in the M_r 21,500 - 66,000 range (possibly fragments of alpha-amylase) was also seen. IEF immunoblots showed that the polyclonal antisera recognised both the high and low pI isozymes; the reaction of the antiserum to high pI amylases was somwhat less intense with the low pI isozymes

than the corresponding reaction of the high/low pI amylase antibody. Even though the wheat monoclonal antibodies were produced by immunizing mice with a mixture of both high and low pI isozymes of amylase, only one of the six antibodies (10581) detected both groups of isozymes; the remaining ones bound the high pI group only.

Example 2: Characterisation of antibody performance in ELISAs for alpha-amylase in wheat

Indirect and Direct ELISAs

Antibodies were purified using either Gamma Bind (Pharmacia) Protein G affinity chromatography (IgG isotypes) or ammonium sulfate preciptation (IgM isotypes). For use in direct ELISA, epitope mapping and as detecting conjugates in two-site ELISA, antibodies were coupled to horseradish peroxidase (Boehringer-Mannheim, Germany) using a method modified from Nakane, P.K.; and Kawaoi, A. (Journal of Histochemistry and Cytochemistry 22 (1974) 1084-1091). Antibodies were initially titrated by indirect and direct ELISA, before being evaluated for their ability to capture and detect alpha- amylase in sandwich ELISA. In the indirect and direct assays, 96 well plates (Nunc Maxisorp, Roskilde, Denmark) were coated for 16 h at 20 °C with 100 µL of purified alpha-amylase antigen at 1 µg/well in 50 mM carbonate buffer, pH 9.6. Wells were then washed three times with PBS - 0.05 % Tween 20 (PBST), and non-specific binding sites were blocked with 1 % BSA in PBS for 1 h at room temperature. Microwell-bound antigen was incubated for 90 min with 100 µL of antibody solution diluted in 1 % BSA in PBS and then washed three times with PBST. This was followed by a 30 min incubation with either 100 µL/well peroxidase-labelled rabbit anti-mouse or goat anti-rabbit immuno-globulins (DAKO, Glostrup, Denmark)

diluted 1:2000 and 1:400 respectively in 1 % BSA in PBS. After four washes, 150 μL substrate-chromogen (2 mM 2,2'-azino-bis-3-ethylbenzthiazoline sulfonic acid (Sigma) in 0.1 M sodium citrate buffer, pH 4.5, containing 0.003 % H_2O_2 (ABTS)), was added and plates incubated for 20 min at room temperature. The enzyme reaction was terminated by the addition of 50 μL oxalic acid (3 %, w/v), and absorbance values were measured at 414 nm. Titration against purified alpha-amylase using indirect ELISA (Figure 1) indicated that each of all the antibodies detected alpha-amylase with high sensitivity in this assay format.

Sandwich ELISAs

For sandwich ELISA, plates were coated for 16 h at 20 °C with 100 μL of either purified monoclonal antibodies or polyclonal antibodies at 1μg /well in 50mM carbonate buffer, pH 9.6. All subsequent steps were carried out at 20 °C. The wells were washed 3 times with PBST and non-specific binding sites blocked with 1 % BSA in PBS for 1 h. Purified alpha-amylase was serially diluted in 1 % BSA in PBST, added to the wells (100 μl/well), and incubated for 1 h. After washing 3 times with PBST, 100 μL of HRP-labeled monoclonal antibody or polyclonal antibody diluted in 1 % BSA in PBST was added to all wells and incubated for 30 min. The dilution of labeled antibody used had been previously determined by direct ELISA to provide an absorbance of between 1.0 and 1.5. Plates were washed as before and ABTS substrate was added to all wells. The reaction was stopped after 20 min and absorbance values were measured at 415 nm. Samples were analyzed in triplicate and the absorbance of blank wells (no addition

of alpha-amylase) was subtracted from the absorbance of each well.

All possible combinations of monoclonal and polyclonal antibodies were tested initially as capture and/or detection antibodies in the plate sandwich ELISA, using a wide range of concentrations (0.001-10 ug/mL) of purified amylase. Each polyclonal antibody functioned well as either a capture or detection antibody when used in conjunction with either the same antibody or another polyclonal antibody. Alpha-amylase was sensitively detected with a limit of detection (absorbance of 0.1 above background) of about 1 ng/mL, for either high pI amylase or high/low pI amylase. One monoclonal antibody (185612) also functioned as either a capture or detection antibody when used in conjunction with a polyclonal antibody (Figure 2). However, the other monoclonal antibodies did not function as either capture or detection reagents; i.e. there was no difference between the absorbance of wells to which amylase had been added and the blank wells which contained no enzyme. Similarly negative results were obtained when these monoclonal antibodies were immobilised through rabbit immunoglobulins to mouse immunoglobulins rather than by direct adsorption to the solid phase. Thus, although the monoclonal antibodies were specific for alpha-amylase on immunoblots and sensitively detected the enzyme in indirect ELISAs, they failed to detect amylase in a plate sandwich ELISA. Use of higher antibody coating levels (up to 10 µg/ well) and different coating buffers (e.g. phosphate-buffered saline, pH 7.2) did not effect antibody performance. A similar pattern of antibody performance was also noted in the rapid tube ELISA (Example 3) and immunochromatography formats (Example 4).

Characterisation of the epitopes recognised by anti-amylase antibodies

The ability of only one of the monoclonal antibodies (185612) to detect alpha-amylases in the sandwich ELISA format as either capture or detection antibody raised the possibility that it may recognise an epitope in the amylase sequence that has a distinctive sequence and other properties from the epitopes in the other monoclonal antibodies. Firstly, to identify the linear epitopes recognised by the antibodies, a series of decapeptides with pentamer overlaps was synthesised corresponding to the entire coding region of the high pI alpha-amylase from wheat (clone amy 1/13 of Baulcombe, D.C.; Huttly, A.; Martienssen, R.A.; Barker, R.A.; Jarvis, M.G. Molecular and General Genetics 209 (1987) 33-40). This is a representative of the isozyme family which is preferentially expressed in wheat grain during germination (Lazarus, C.M.; Baulcombe, D.C.; Martienssen, R.A. Plant Molecular Biology 5 (1985) 13-24; Cejudo, F.J.; Cubo, M.T.; Baulcombe, D.C. Plant Science 106 (1995) 207-213). The decapeptides were prepared by solid-phase peptide synthesis on the tips of primed pins in an 8x12 array for direct testing in microwell plates, and the binding of five monoclonal nd two polyclonal antibodies analysed using indirect ELISA. Non-specific antibody binding to the pins was blocked by incubation for 1 hour at 20 °C in 2 % BSA in 0.15 M NaCl - 10 mM sodium phosphate, pH 7.2 containing 0.05 % Tween 20. The pins were transferred to wells containing peroxidase-labelled antibodies (diluted to provide a maximal abosrbance of about 1.0) in 2 % BSA in 0.15 M NaCl - 10 mM sodium phosphate, pH 7.2 containing 0.05 % Tween 20 and incubated for 1 hour at 20° C with shaking, before being washed four times in 0.15 M NaCl -

10 mM sodium phosphate, pH 7.2. Antibody binding to specific peptides was revealed by incubation in 2 mM diammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) - 0.003 % (w/v) hydrogen peroxide in 50 mM sodium citrate, pH 4.5 for 40 minutes at 20 °C. Replicate assays were performed to ensure that consistent results were obtained.

The antibodies strongly bound only a small number of peptide sequences in the high pI alpha-amylase sequence (Figure 3). Antibody 185612, which functions as either capture or detection antibody in two-site assays, detected three main sequence regions. It most strongly recognised two arginine-rich sequences, which also contain other charged amino acids, especially aspartate in the case of the second amino sequence: IDRLVSIRTRGQIHS and CRDDRPYADG. In addition, a valine-rich peptide was detected: VNWVNKVGGS. A polyclonal antiserum to high pI alpha-amylase (ALI) also detected the IDRLVSIRTRGQIHS and VNWVNKVGGS sequences, as well as several other epitopes, in keeping with the polyclonal nature of the antiserum. A second polyclonal antiserum (ADL), prepared to a mixture of high and low pI alpha-amylases, also detected the VNWVNKVGGS sequence, along with several others (Figure 3). Four monoclonal antibodies which sensitively detected alpha-amylase in indirect or direct ELISA but not two-site assays, detected epitopes that were distinct from those recognised by antibody 185612 (Figure 3). In some cases the polyclonal antisera also recognised some of the same epitopes, suggesting that there are immunodominant regions in the alpha-amylase sequence. One antibody, 10413, recognised a peptide (KVGGSGPGTT) (SEQ ID NO: 5) that had a partial overlap with the valine-rich peptide recognised by antibody 185612 and the polyclonal antisera

(VNWVNKVGGS). However, since 10413 did not bind to VNWVNKVGGS its epitope is clearly also distinct. Thus, these results indicate that the antibodies which function in the two-site assay recognise distinct epitopes from those recognised by other antibodies specific for alpha-amylase, but which do not function in the two-site assay.

In order to assess whether there was also a difference in the conformational nature between the epitopes recognised by the antibodies which function in the two-site assay and other antibodies to alpha-amylase, effect on antibody binding of partial denaturation with urea of the alpha-amylase antigen were studied. High pI alpha-amylase was dissolved in either: 1. PBS (phosphate-buffered saline, 50 mM sodium phosphate - 150 mM NaCl, pH 7.2); 2. PBS - 1 % (w/v) dithiothreitol; 3. 8 M urea; 4. 8 M urea - 1 % (v/v) dithiothreitol, at a series of concentrations ranging from 0.01 μg/mL to 10 μg/mL. The antigens were coated onto microwell ELISA plates for 16 hours at 20-23 °C and binding to the PBS-dissolved "native" and treated amylases assessed by indirect ELISA. While each antibody detected native amylase, there was a clear difference in behaviour between different antibodies. The antibodies which functioned in two-site assays (185612 and the polyclonal antisera) bound significantly more poorly to urea-denatured amylase, while the binding of each of the four antibodies that did not function in the two-site assays was unaffected. Reduction with dithiothreitol did not similarly decrease antibody binding. Results with 185612, 15764 and 15689 are shown in Figure 4. These results provide further evidence that the epitopes recognised by antibodies which functioned in two-site assays had particular and distinct properties and that the

conformation of such epitopes was affected by partial denaturation with urea.

Example 3: Rapid tube sandwich ELISA for detection of Alpha-Amylase in preharvest-sprouted wheat

Based on the initial performance of the antibodies in the plate sandwich ELISA, rapid tube ELISAs were developed for detection of alpha-amylase. The larger volume of the tubes makes them more suited for field use, since reagents can be added using droppers, and larger reagent volumes can be used, making the assay more acceptable to non-laboratory personnel. Sets of polystyrene test tubes (12 mm diameter x 75 mm long) were coated for 16 h at 20°C with purified capture antibody (5 µg in 500 µL 50 mM sodium carbonate buffer, pH 9.6). Tubes were washed 3 times with PBST, and non-specific binding blocked for 60 min using 1 % BSA in PBS. Large batches of tubes could be prepared by freeze drying the antibodies onto the tube surface. The conventional ELISA assay format involving sequential addition of wheat extract and enzyme conjugate, with an intermediate washing step, was simplified to provide an assay protocol involving simultaneous addition of wheat extract and enzyme (with no intermediate washing step). In this assay, antibody HRP conjugates in 1 % BSA in PBST were added (50 µL/tube) followed immediately by the addition of 500 µL of undiluted grain extract (prepared in 85 mM NaCl), and incubated for 5-10 min. The initial procedure for extraction of amylase (i.e. blending whole grain in sodium malate buffer) could be simplified by vortex mixing wholemeal flour (ground in the Jupiter mill) for 4 min in malate buffer, and the malate extraction buffer could also be replaced by a simple salt solution (Table II). The use

of any of 3 extraction solutions (85 mM or 50 mM NaCl or 50 mM NaCl plus 20 mM $CaCl_2$) provided adequate extraction of enzyme and discrimination between sprouted and sound wheat. Thus an extraction buffer consisting of 85 mM NaCl (which can be made easily under field conditions by dissolving a NaCl tablet in a pre-determined volume of water) was used in subsequent experiments; this may also remove effects of variation in water quality in remote situations. Tubes were washed 3 times with 85 mM NaCl and 500 µL of substrate-chromogen (0.6 mg/mL 3,3',5,5'-tetramethylbenzidine in 0.1 M sodium acetate, pH 5 containing hydrogen peroxide) was added to each tube. The reaction was stopped after 3-5 min by the addition of 250 µL of 1.25 M sulfuric acid. The absorbance was measured at 450 nm using an RPA-1 Rapid Photometric analyser (Source Scientific, Garden City, CA, USA).

The tube assays were less sensitive than the plate assays with detection limits of approximately 4 ng/mL amylase, probably due to the much shorter incubation periods used. With an initial set of 10 wholemeals, mean ELISA absorbance (colour development) showed a decrease with increasing Falling Number for the tube assay in either format. The results of a comparison in which enzyme was extracted from wholemeal flour in 85 mM NaCl buffer either by intermittent vortex mixing for 4 min, or by wrist-action shaking for 15 sec, showed that hand shaking not only allowed for sufficient extraction of amylase for discrimination between sprouted and sound wheat, it also removed the need for vortex mixing, thus simplifying the tube assay proceedure further.

The performance of this assay was tested with several sets of naturally-sprouted grain samples, with Falling Numbers ranging from 62 to 494. This included 56

samples from Western Australia (1995 harvest), 30 samples from the 1995 harvest in New South Wales, Australia (Suneca, Hartog, Sunstate and Janz), 130 samples comprising 8 cultivars (Hartog, Cunningham, Pelsart, Banks, Sunco, Sunstate, Perouse and Janz), from Queensland, Australia (1996 harvest) and 108 samples comprising 6 cultivars (Hartog, Halberd, Sunland, Tincurrin, Matong and Vulcan) from New South Wales, Australia and subjected to controlled wetting. These varieties contained hard and soft wheat types of diverse protein contents (8-15 % protein) and end-use types. Falling Numbers for wholemeal (ground with a Falling Number 3100 mill) were determined in duplicate. The analyses were performed with the aim of establishing the relationship between Falling Number and colour development in the ELISA test, as well as establishing whether the relationships noted were substantially independent of the wheat variety analyzed.

The results of these analyses, shown in Figures 5A and B, indicated that relative ELISA colour development was strongly (and negatively) correlated with Falling Number for both tube assays. Wheats with Falling Numbers below 350 seconds could be discriminated from sound wheats, with decreasing Falling Numbers producing increasing assay colour.

The results of the ELISA also correlated with alpha-amylase activity measurements, determined using the Ceralpha assay (Megazyme International, Deltagen, Melbourne, Australia), which utilizes p-nitrophenylheptanoside as substrate (McCleary, B.V.; and Sheehan, H. Journal of Cereal Science 6 (1987) 237-251). For the 130 Queensland samples, the correlation between Ceralpha enzyme activity (units/g flour) and Falling Number was r = 0.93. Although modifications made to the

enzyme assay have increased its sensitivity, it still appeared to be less sensitive than the Falling Number test or the tube ELISA, by failing to clearly discriminate wheat samples with Falling Numbers greater than 250 seconds. Enzymes such as amylases can also be quantified directly by various enzyme assays. The tube ELISA we have developed, showed a strong negative correlation with Falling Number (for 3 diverse sets of wheats) and a positive correlation with the Ceralpha enzyme assay. The ELISA is simple and easy to use, and is reproducible over a wide range of Falling Numbers. Although the tube sandwich ELISA was less sensitive than the plate ELISA, it still had the capacity to detect Falling Numbers below 350 sec (the critical industry cut off point). Above 350 seconds, it has been shown by others that it is difficult to establish close correlations between Falling Number and alpha-amylase activity (Mares, 1989). Analysis of three sets of wheat samples from different environments demonstrated that the relationship between ELISA absorbance and Falling Number had little dependence on wheat variety. The precision of sample analysis using the field ELISA was similar to the precision of the Falling Number test.

Example 4: Immunochromatography assay for alpha-amylase

Immunochromatography (IC), in which bound and unbound components are separated by capillary flow rather than a washing step, potentially provides an even simpler test format that the coated-tube ELISA. Several patents and publications teach the principles of this assay format (Birnbaum, S.; Uden, C.; Magnusson, C.G.M.; Nilsson, S. Analytical Biochemistry 206 (1992) 168-171; Ching, S.; Billing, P.; Gordon, J. United States Patent No. 5,120,643, issued June 9, 1992; Lou, S.C.; Patel, C.;

Ching, S.; Gordon, J. Clinical Chemistry 39 (1993) 619-624) and it has been widely applied to the "yes-no" analysis of medical analytes such as urinary human chorionic gonadotrophin analysis in urine for pregnancy testing, and screening for infectious diseases such as malaria. The format has, however, had little application to agricultural analytes.

In the IC format used, a complex of antigen and labelled antibody is allowed to form then migrate by capillary action up a porous test strip. The complex is captured by a band of immobilised antibody on the strip. In the test format used, the wheat grain sample was ground to a fine meal, and 0.5 g meal is shaken with 6 mL of 85 mM NaCl solution in a tube provided. Two drops (60 µL) of the grain extract are added to a sample pad where, if alpha-amylase is present, it binds to an antibody attached to visible (maroon) colloidal gold. A second antibody to alpha-amylase is immobilised as a line across the test strip. After addition of PBS - 0.05 % Tween 20 wetting agent to the sample pad, the colloidal gold and complexes of colloidal gold and alpha-amylase migrate up the test strip crossing the second antibody line across the test strip. If the sample has a Falling Number below 350 (i.e. contains significant amounts of alpha-amylase), the gold-amylase complex will be captured by the antibody on the membrane and a pink-maroon test line forms. In a negative sample, no test line forms. The card also contains a "control line" of immobilised goat anti-mouse or goat anti-rabbit immunoglobulin antibodies, which provides a positive pink-maroon result in every test.

The different antibody combinations were tested for performance in the IC assay, and only those combinations which functioned in a double-antibody sandwich ELISA (i.e.

Mab 185612 and the two polyclonal antibodies) also functioned in the double antibody IC test; antibodies 10413, 15689, 15724 and 15764 did not. Although IC is commonly regarded as a qualitative test format, we found that the intensity of the colour developed in the test line depended upon the concentration of alpha-amylase in the test sample and thus the extent of the weather damage in the sample, such that more colour indicates greater weather damage. Colourless or very pale results occurred when the sample was sound (Falling Number over 350 seconds). The level of weather damage and likely Falling Number for the sample can be determined by analysing some samples of known Falling Number and by comparing test results (Figure 6). Test results were also reproducible between and within assays (Figure 7). Results can either be read with respect to standards of known Falling Number, for example on a colour card by using a reflectometer using either white light illumination or light-emitting diode illumination. When manually reading the results of the tests, a 5 min assay time is suitable. It is be possible to further decrease this time in conjunction with a reflectometer.

These assays (in the form of a simple kit) have the potential for on-farm use by individual growers allowing identification of areas of sprouting prior to harvest, thus preventing contamination of sound wheat by wheat that is weather damaged. Currently growers may harvest grain across their whole property, and tests on elevator receival are done on the whole parcel of grain. However, except in very wet harvests, the extent and presence of preharvest sprouting can vary quite markedly between and within fields, being dependant on the rate of drying of the crop after rainfall has occured (affected by field aspect and drainage), wheat variety sown and time of

sowing. Growers usually have an intimate knowledge of the behaviour of different parts of their own property, and if they were able to test the grain from different paddocks and parts of paddocks before harvest, it should be possible to harvest the damaged grain separately from sound grain and avoid the financial losses that result from downgrading the whole crop.

The two-site immunoassays of the present invention enable the simple assessment of the level of alpha-amylase and thus the likely Falling Number of the grain sample. This is of particular advantage since the two-site immunoassays can be applied at mill or silo (elevator) receival of grain or could be used on farms with minimal equipment requirements.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said immunoassay comprising;

(i) exposing said test sample to a first antibody or fragment thereof which specifically or preferentially binds to a first epitope on said alpha-amylase, under conditions permitting binding of said first antibody or fragment thereof to alpha-amylase if present,

(ii) subsequently exposing bound alpha-amylase, if any, to a second antibody or fragment thereof which specifically or preferentially binds to a second epitope on said alpha-amylase that is distinct from said first epitope, under conditions permitting binding of said second antibody or fragment thereof to said bound alpha-amylase, and

(iii) detecting any binding of said second antibody or fragment thereof to said bound alpha-amylase,

wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80% sequence identity.

2. An immunoassay according to claim 1, wherein either of said first or second epitopes is an epitope comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 90% sequence identity.

3. An immunoassay according to claim 1, wherein either of said first or second epitopes is a conformational epitope comprising one or more of the amino acid

sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3).

4. An immunoassay according to claim 1, wherein either of said first or second epitopes is a conformational epitope comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3).

5. An immunoassay according to any one of the preceding claims, wherein said first antibody or fragment thereof or said second antibody or fragment thereof is provided bound to a solid support.

6. An immunoassay according to claim 5, wherein the solid support is selected from microwell plates, membranes, beads, particles, sensors and porous test strips.

7. An immunoassay according to any one of the preceding claims, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of a readily detectable label.

8. An immunoassay according to claim 7, wherein the detectable label is selected from detectable enzymes, radioisotopes, luminescent labels and fluorescent labels.

9. An immunoassay according to any one of claims 1 to 6, wherein binding of the second antibody or fragment thereof to alpha-amylase is detected through the use of immunochromatography or agglutination.

10. Am immunoassay according to any one of the preceding claims, wherein at least one of the first and second antibodies or fragments thereof is selected from

monoclonal antibodies or fragments thereof and recombinant antibody fragments.

11. An immunoassay according to any one of the preceding claims, wherein the test sample is obtained from a cereal grain.

12. An immunoassay according to claim 11, wherein the cereal grain is selected from the group consisting of bread wheat (*Triticum aestivum*), durum wheat (*Triticum turgidum* var. durum), club wheat *(Triticum compactus)*, rye (*Secale cereale*), triticale (*Triticosecale* species) and barley (*Hordeum vulgare*).

13. An immunoassay according to claim 11 or 12, wherein the test sample is an aqueous extract from grain, grain meal or flour.

14. An immunoassay according to any one of the preceding claims, wherein said immunoassay provides for the quantitative detection of alpha-amylase by further comprising;

(iv) comparing the level of detected binding of the second antibody or fragment thereof to alpha-amylase against a suitable standard.

15. An immunoassay according to any one of the preceding claims when used to detect weather damage in a cereal grain.

16. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2),

VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 80% sequence identity.

17. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically binds to an epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3) and variants thereof showing ≥ 90% sequence identity.

18. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to a conformational epitope on alpha-amylase comprising one or more of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3).

19. A monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner which specifically or preferentially binds to conformational epitope on alpha-amylase comprising all of the amino acid sequences; IDRLVSIRTRGQIHS (SEQ ID NO: 1), CRDDRPYADG (SEQ ID NO: 2), VNWVNKVGGS (SEQ ID NO: 3).

20. A kit for performing a two-site immunoassay for the qualitative or quantitative detection of alpha-amylase in a test sample, said kit comprising a container or solid support including a monoclonal antibody or fragment thereof, recombinant antibody or fragment thereof, recombinant antibody fragment or binding partner according to any one of claims 16 to 19.

21. A kit according to claim 20, further comprising a container including an aqueous extraction agent for extracting alpha-amylase from grain, grain meal or flour.

22. A kit according to claim 20, wherein the extraction agent is aqueous NaCl or $CaCl_2$.

FIGURE 1 1/10

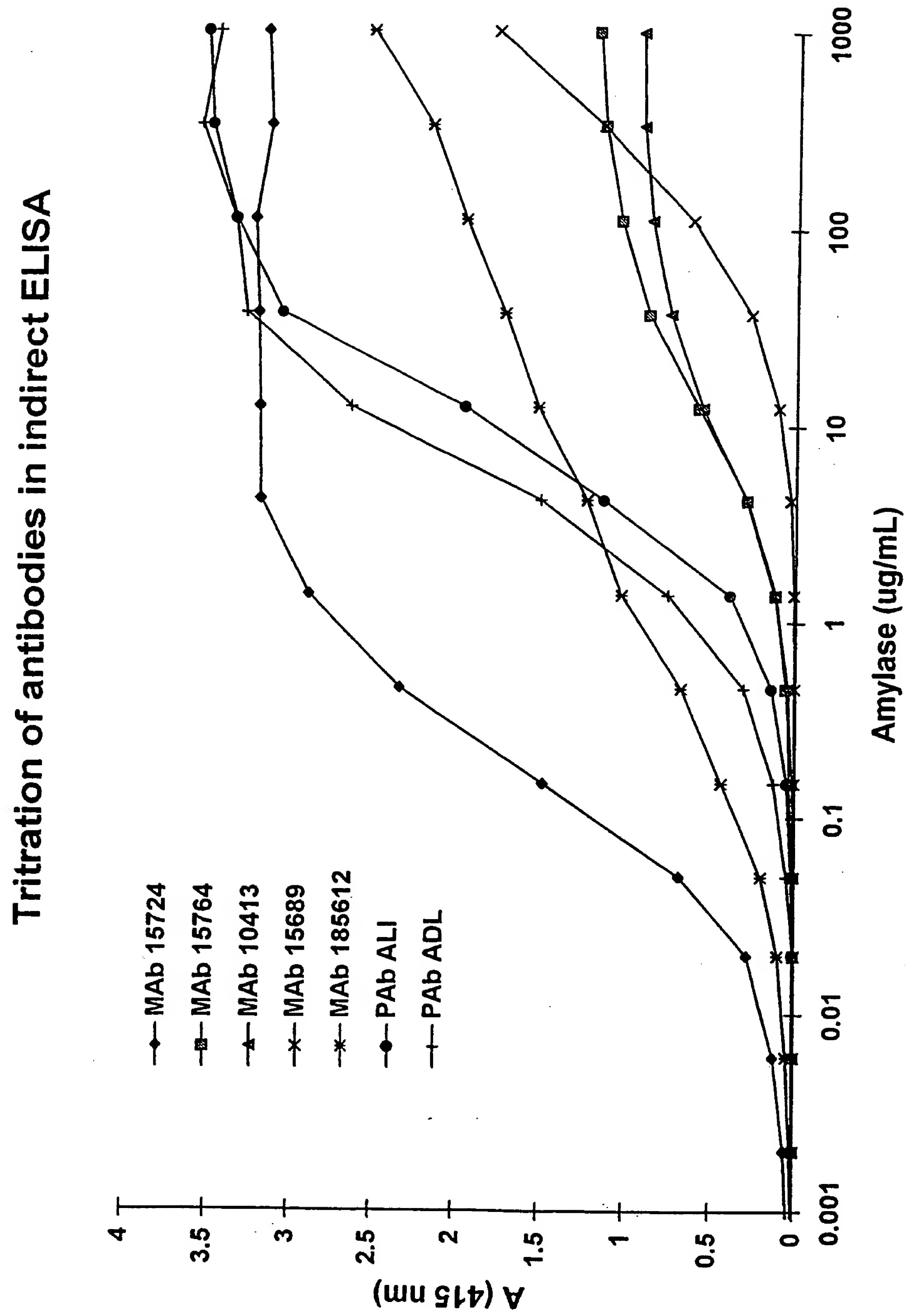

Tritration of antibodies in indirect ELISA
MAb 15724
MAb 15764
MAb 10413
MAb 15689
MAb 185612
PAb ALI
PAb ADL
A (415 nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
0.001
0.01
0.1
1
10
100
1000
Amylase (ug/mL)

FIGURE 2

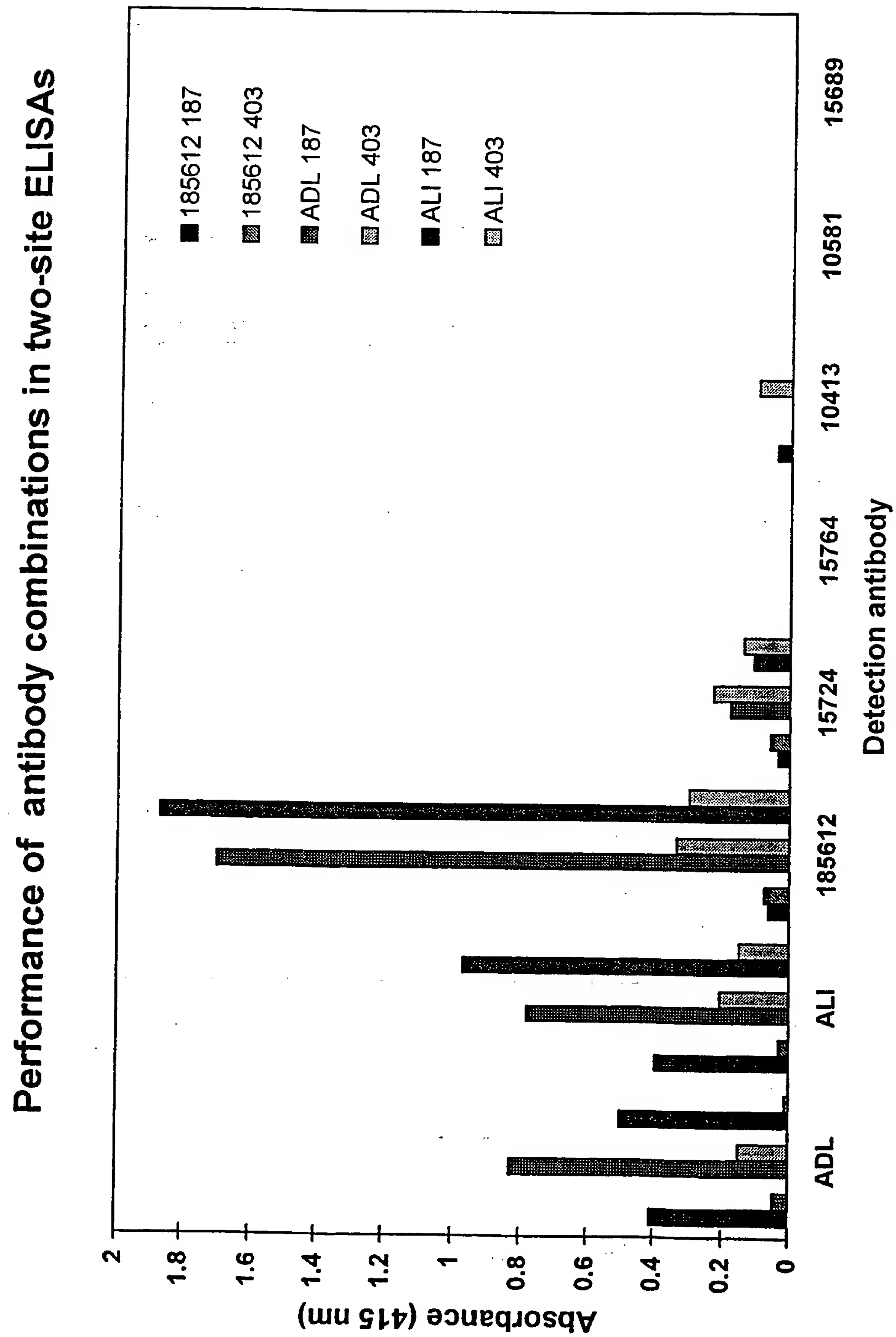

Performance of antibody combinations in two-site ELISAs
185612 187
185612 403
ADL 187
ADL 403
ALI 187
ALI 403
Absorbance (415 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ADL
ALI
185612
15724
15764
10413
10581
15689
Detection antibody

FIGURE 3


```
amy 1/13  MASKHLSLFLVLLGLSASLASGQVLFQGFNWESWKHNGGWYNFLMGKVDDIAAAGVTHVWLPPASQSVSEQGYMPGRLYDLDASKYGNKAQLKSLIGALH
185612
ADL                                                                                  ----------
ALI                                                                   ---------------
10413
15689
15724                                ----------
15764                                                                                          ----------

amy 1/13  GKGVKAIADIVINHRTAERKDGRGIYCIFEGGTPDARLDWGPHMICRDDRPYADGTGNPDTGADFGAAPDIDHLNPRVQKELVELLNWLRTDIGFDGWRF
185612                                                   -----
ADL                                  ---------------          ------------------------------
ALI                                       ----------                                ----------
10413     ----------
15689                                ----------
15724                                                                                                    ----------
15764                                ----------               ----------                     --------------------

amy 1/13  DFAKGYSADVAKIYVDRSEASFAVAEIWTSLAYGGDGKPNLNQDPHRQELVNWVNKVGGSGPGTTFDFTTKGILNVAVEGELWRLRGTDGKAPGMIGWWP
185612.                                                       ----------
ADL                                                           ----------
ALI                                                           ----------
10413                                                              ----------                     ----------
15689
15724     -----                                                              ----------
15764

amy 1/13  AKAVTFVDNHDTGSTQHMWPFPSDRVMQGYAYILTHPGPPCIFYDHFFDWGLKEEIDRLVSIRTRQGIHSESKLQIIEADADLYLAEIDGKVIVKLGPRY
185612                                                             ---------------
ADL
ALI                                                                     ----------
10413
15689                                                                                ---------------
15764                                                                                ---------------

amy 1/13  DVGHLIPGGLKVAAHGKDYAIWEKI (SEQ ID NO: 4)
185612
ADL       ----------
ALI
10413
15689          ----------
15764
```

FIGURE 4a

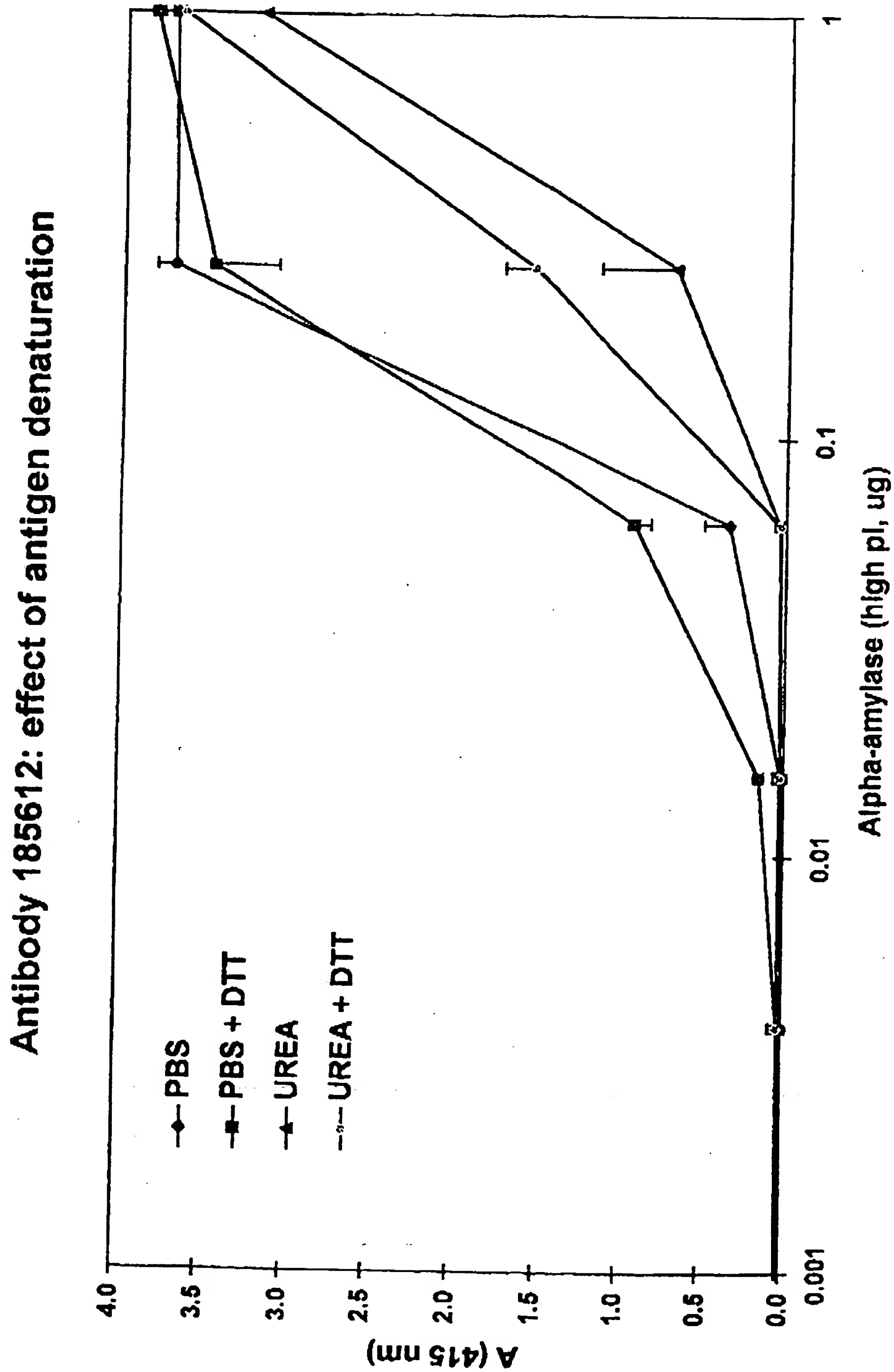

Antibody 185612: effect of antigen denaturation
PBS
PBS + DTT
UREA
UREA + DTT
A (415 nm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
Alpha-amylase (high pI, ug)

FIGURE 4b

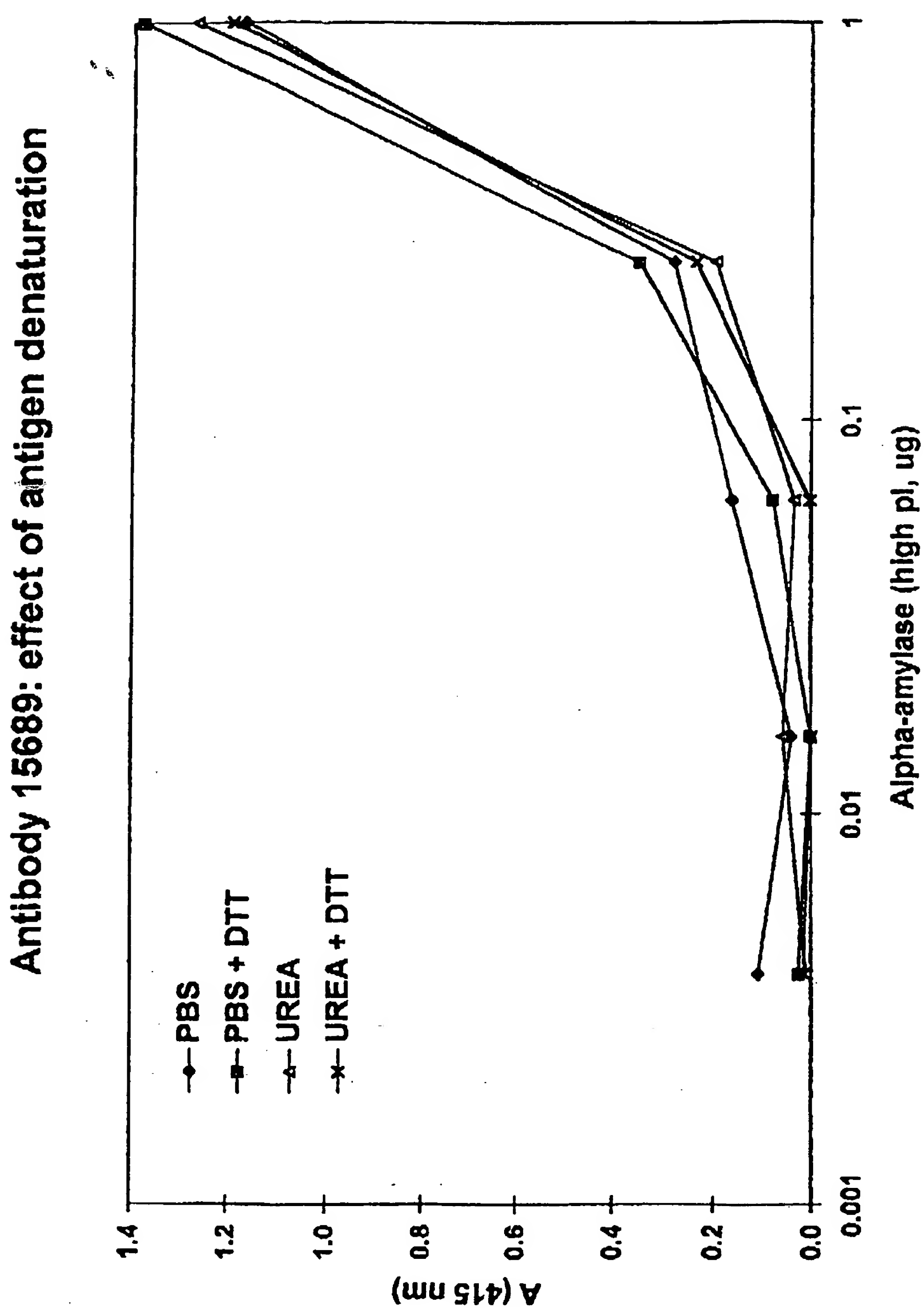

Antibody 15689: effect of antigen denaturation
PBS
PBS + DTT
UREA
UREA + DTT
A (415 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.001
0.01
0.1
1
Alpha-amylase (high pI, ug)

FIGURE 4c

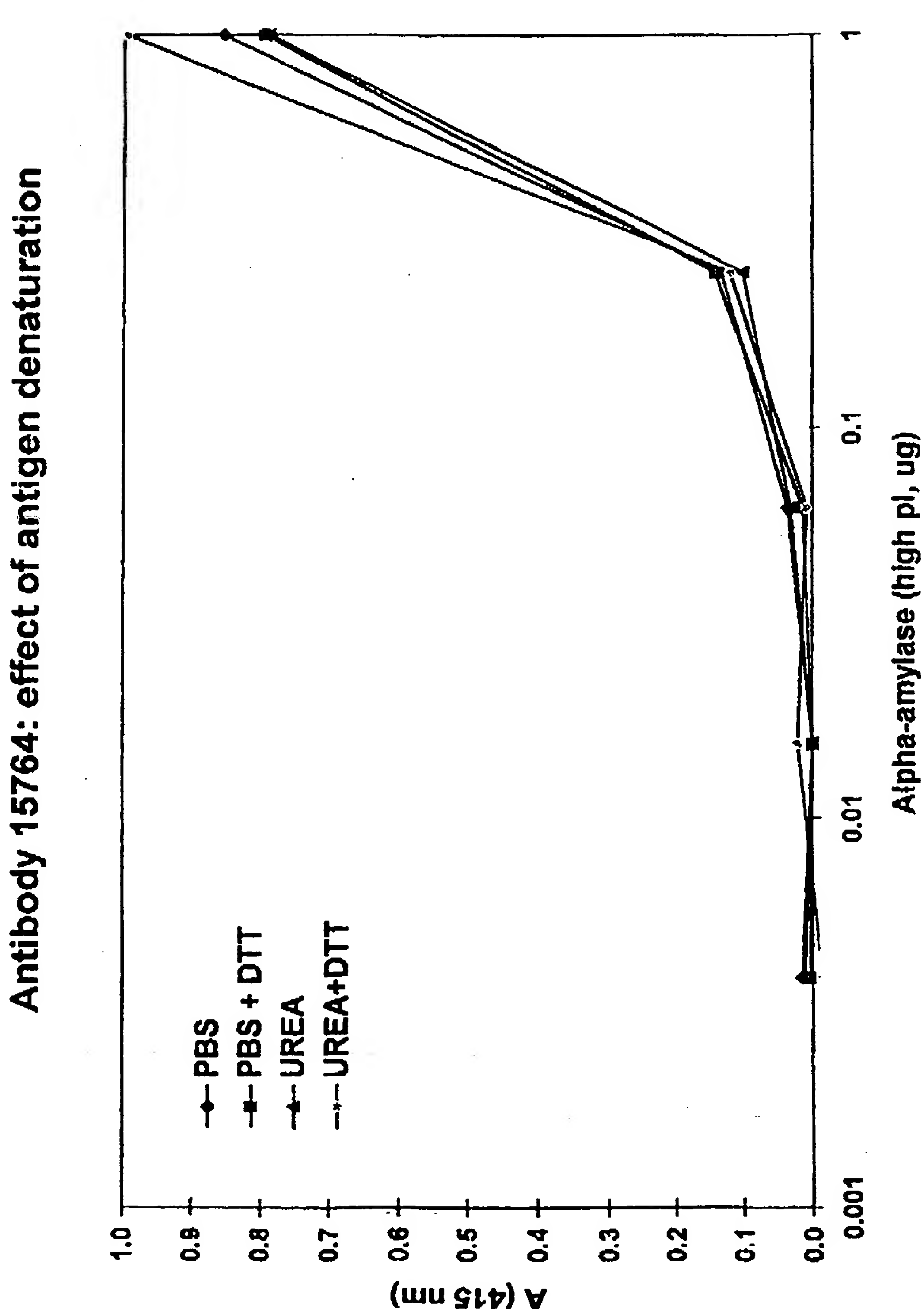

Antibody 15764: effect of antigen denaturation
PBS
PBS + DTT
UREA
UREA+DTT
A (415 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.001
0.01
0.1
1
Alpha-amylase (high pI, ug)

FIGURE 5a

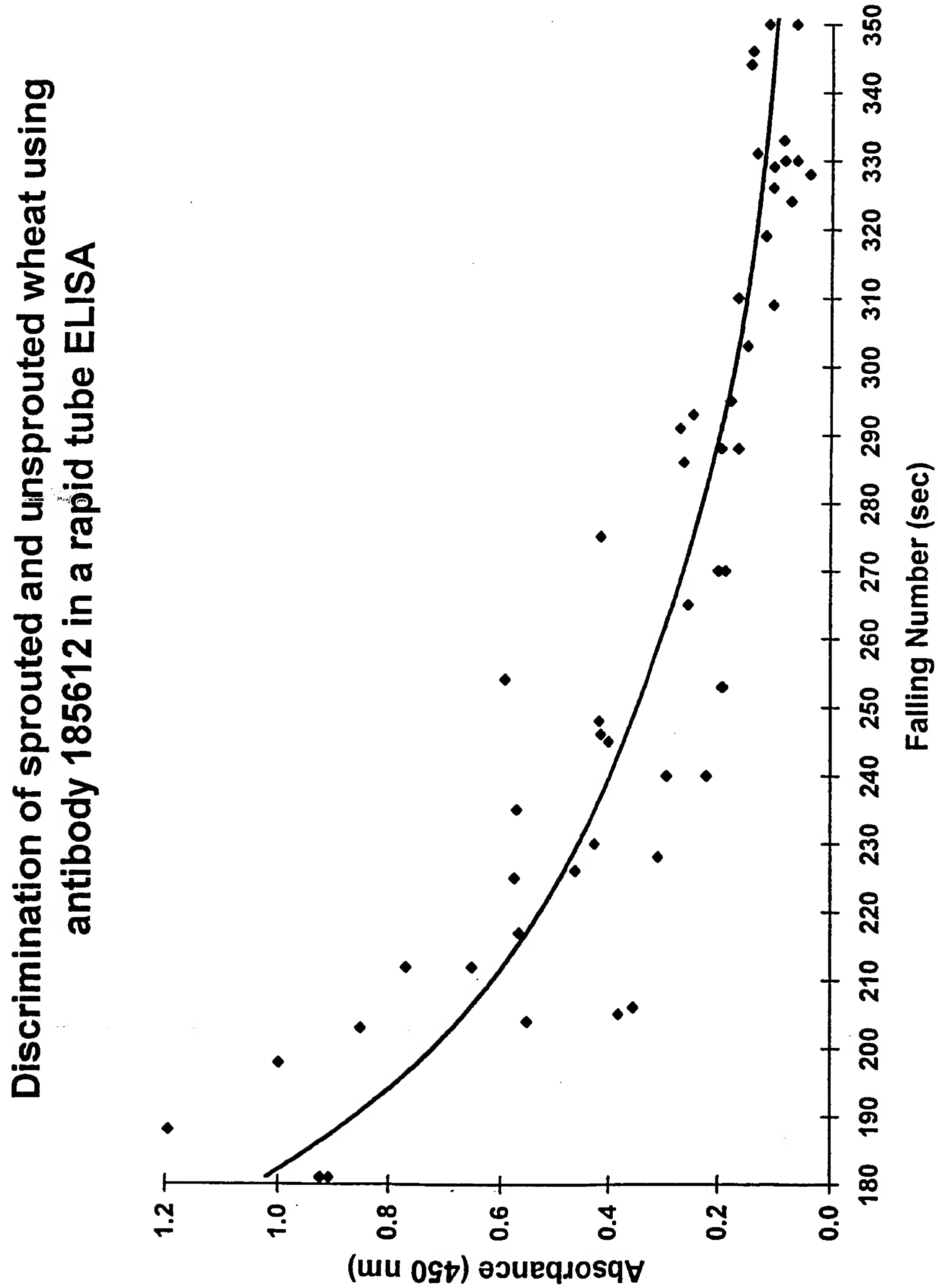

Discrimination of sprouted and unsprouted wheat using antibody 185612 in a rapid tube ELISA
Absorbance (450 nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Falling Number (sec)
180
190
200
210
220
230
240
250
260
270
280
290
300
310
320
330
340
350

FIGURE 5b

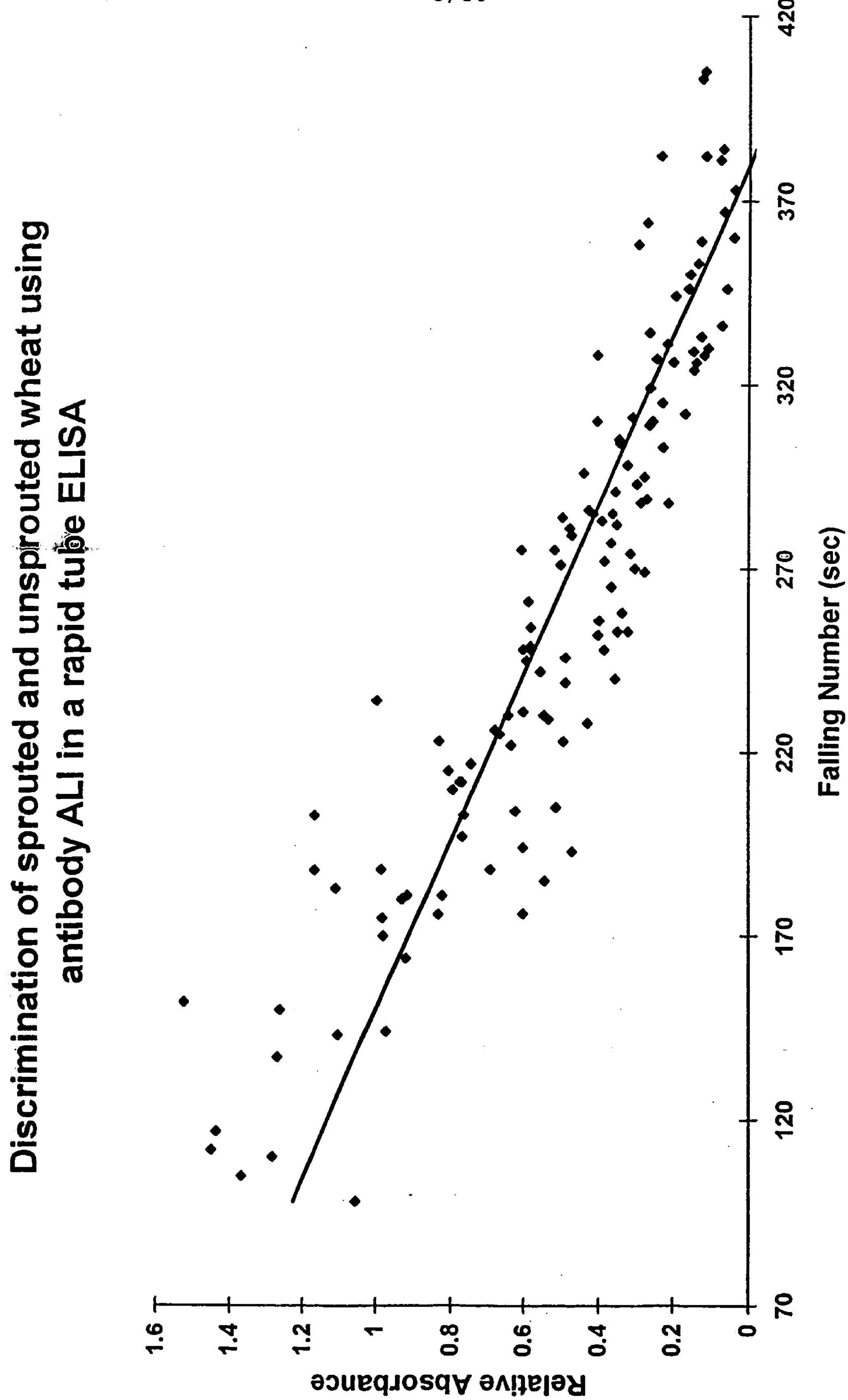
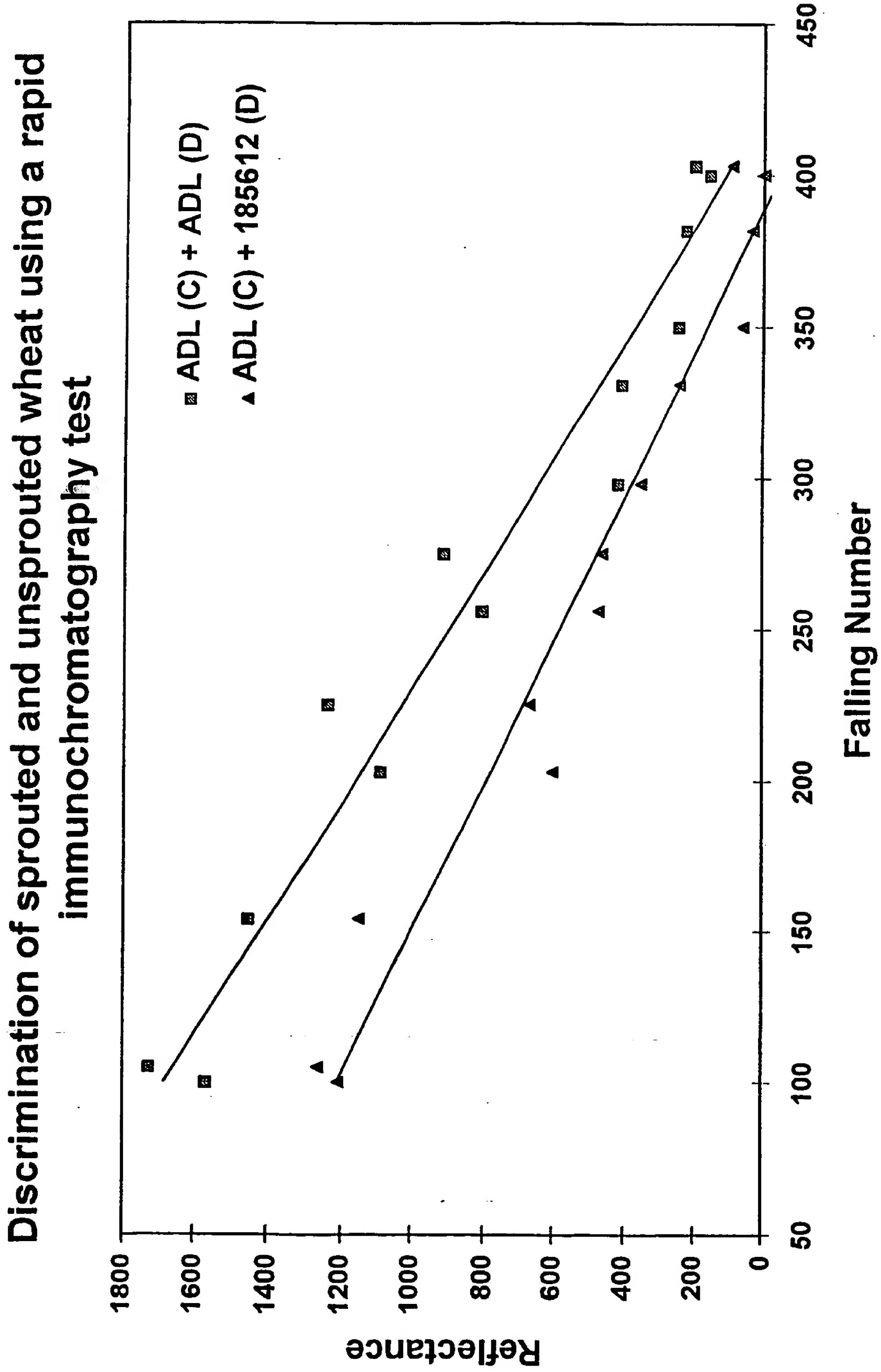
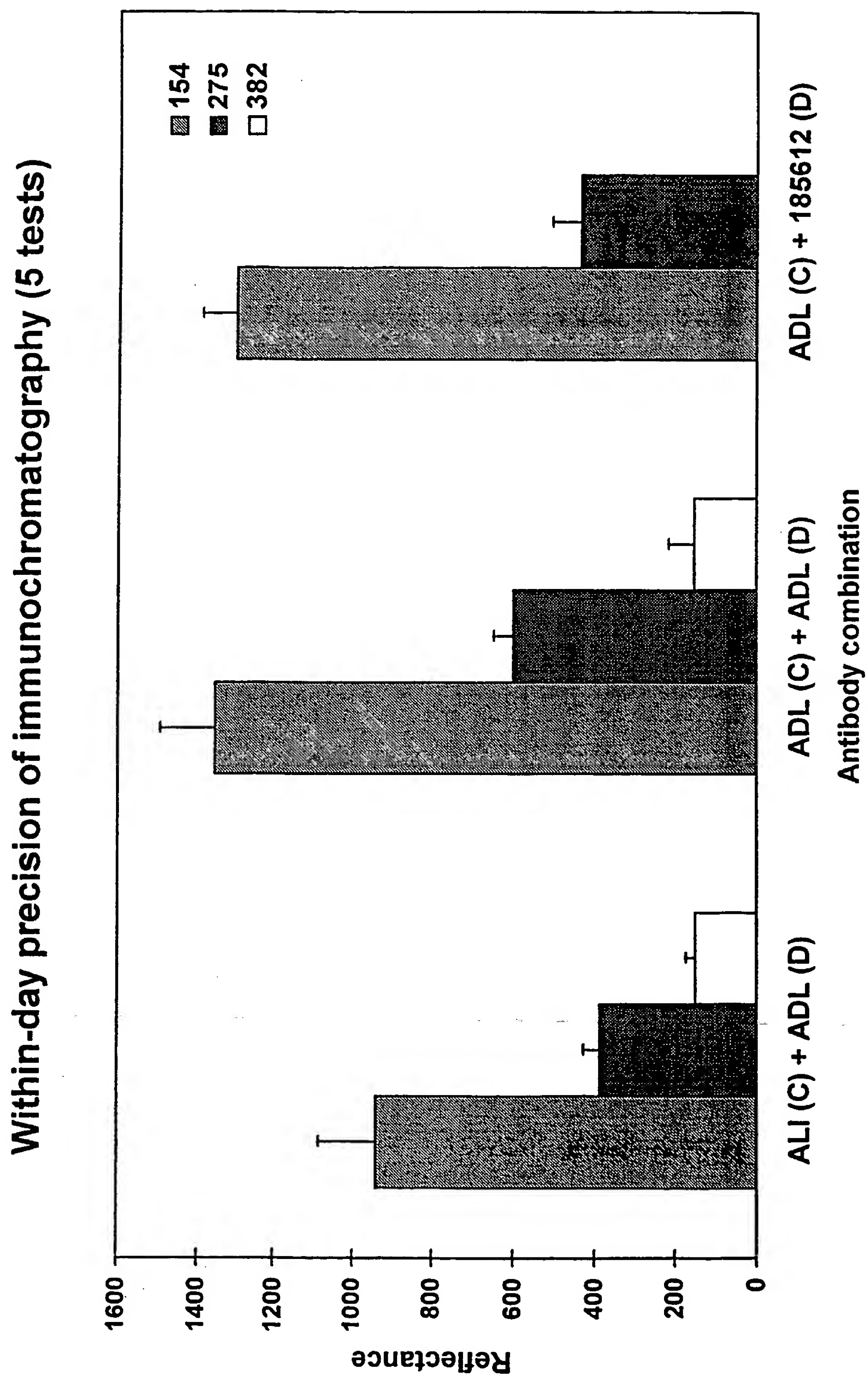

Discrimination of sprouted and unsprouted wheat using antibody ALI in a rapid tube ELISA
Relative Absorbance
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Falling Number (sec)
70
120
170
220
270
320
370
420

FIGURE 6



Discrimination of sprouted and unsprouted wheat using a rapid immunochromatography test
ADL (C) + ADL (D)
ADL (C) + 185612 (D)
Reflectance
1800
1600
1400
1200
1000
800
600
400
200
0
Falling Number
50
100
150
200
250
300
350
400
450

FIGURE 7


Within-day precision of immunochromatography (5 tests)
154
275
382
Reflectance
1600
1400
1200
1000
800
600
400
200
0
ALI (C) + ADL (D)
ADL (C) + ADL (D)
ADL (C) + 185612 (D)
Antibody combination

Sequence Listing:

Applicant: Quality Wheat CRC Limited

Title of the Invention: Detection of preharvest sprouting in cereal grains

Number of SEQ ID NOs: 5

Software: PatentIn Ver. 2.1

SEQ ID NO: 1
Length: 15
Type: PRT
Organism: Triticum aestivum

Sequence: 1

```
Ile Asp Arg Leu Val Ser Ile Arg Thr Arg Gly Gln Ile His Ser
  1               5                  10                  15
```

SEQ ID NO: 2
Length: 10
Type: PRT
Organism: Triticum aestivum

Sequence: 2

```
Cys Arg Asp Asp Arg Pro Tyr Ala Asp Gly
  1               5                  10
```

SEQ ID NO: 3

Length: 10

Type: PRT

Organism: Triticum aestivum

Sequence: 3

Val Asn Trp Val Asn Lys Val Gly Gly Ser
1 5 10

SEQ ID NO: 4

Length: 425

Type: PRT

Organism: Triticum aestivum

Sequence: 4

Met Ala Ser Lys His Leu Ser Leu Phe Leu Val Leu Leu Gly Leu Ser
1 5 10 15

Ala Ser Leu Ala Ser Gly Gln Val Leu Phe Gln Gly Phe Asn Trp Glu
20 25 30

Ser Trp Lys His Asn Gly Gly Trp Tyr Asn Phe Leu Met Gly Lys Val
35 40 45

Asp Asp Ile Ala Ala Ala Gly Val Thr His Val Trp Leu Pro Pro Ala
50 55 60

Ser Gln Ser Val Ser Glu Gln Gly Tyr Met Pro Gly Arg Leu Tyr Asp
65 70 75 80

Leu Asp Ala Ser Lys Tyr Gly Asn Lys Ala Gln Leu Lys Ser Leu Ile
85 90 95

Gly Ala Leu His Gly Lys Gly Val Lys Ala Ile Ala Asp Ile Val Ile
100 105 110

Asn His Arg Thr Ala Glu Arg Lys Asp Gly Arg Gly Ile Tyr Cys Ile
115 120 125

Phe Glu Gly Gly Thr Pro Asp Ala Arg Leu Asp Trp Gly Pro His Met
130 135 140

Ile Cys Arg Asp Asp Arg Pro Tyr Ala Asp Gly Thr Gly Asn Pro Asp
145 150 155 160

Thr Gly Ala Asp Phe Gly Ala Ala Pro Asp Ile Asp His Leu Asn Pro
165 170 175

Arg Val Gln Lys Glu Leu Val Glu Leu Leu Asn Trp Leu Arg Thr Asp
180 185 190

Ile Gly Phe Asp Gly Trp Arg Phe Asp Phe Ala Lys Gly Tyr Ser Ala
195 200 205

Asp Val Ala Lys Ile Tyr Val Asp Arg Ser Glu Ala Ser Phe Ala Val
210 215 220

Ala Glu Ile Trp Thr Ser Leu Ala Tyr Gly Gly Asp Gly Lys Pro Asn
225 230 235 240

Leu Asn Gln Asp Pro His Arg Gln Glu Leu Val Asn Trp Val Asn Lys
245 250 255

Val Gly Gly Ser Gly Pro Gly Thr Thr Phe Asp Phe Thr Thr Lys Gly
260 265 270

Ile Leu Asn Val Ala Val Glu Gly Glu Leu Trp Arg Leu Arg Gly Thr
275 280 285

Asp Gly Lys Ala Pro Gly Met Ile Gly Trp Trp Pro Ala Lys Ala Val
290 295 300

Thr Phe Val Asp Asn His Asp Thr Gly Ser Thr Gln His Met Trp Pro
305 310 315 320

Phe Pro Ser Asp Arg Val Met Gln Gly Tyr Ala Tyr Ile Leu Thr His
325 330 335

Pro Gly Pro Pro Cys Ile Phe Tyr Asp His Phe Phe Asp Trp Gly Leu
340 345 350

Lys Glu Glu Ile Asp Arg Leu Val Ser Ile Arg Thr Arg Gln Gly Ile
355 360 365

His Ser Glu Ser Lys Leu Gln Ile Ile Glu Ala Asp Ala Asp Leu Tyr
370 375 380

Leu Ala Glu Ile Asp Gly Lys Val Ile Val Lys Leu Gly Pro Arg Tyr
385 390 395 400

Asp Val Gly His Leu Ile Pro Gly Gly Leu Lys Val Ala Ala His Gly
405 410 415

Lys Asp Tyr Ala Ile Trp Glu Lys Ile
420 425

SEQ ID NO: 5

Length: 10

Type: PRT

Organism: Triticum aestivum

Sequence: 5

Lys Val Gly Gly Ser Gly Pro Gly Thr Thr
1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00995

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int Cl^6:	G01N 33/10, 33/573, 33/577, C07K 14/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: G01N/IC, C07K/IC, C12P/IC

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, FSTA, AGRICOLA, - KEYWORDS: WHEAT, RYE, BARLEY, TRITICUM, GRAIN, CEREAL, FLOUR, IMMUNOASSAY, MONOCLONAL, ELISA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SANDER et al, "Development of a two site enzyme linked immunosorbent assay for alpha amylase from Aspergillus oryzae based on Monoclonal antibodies", J IMMUNOL. METHODS (1997) vol 210 no 1 pp 93-101 whole document	1-22
Y	LECOMMANDEUR D et al,"Monoclonal antibodies to barely α-amylase I: production, characterization, and application to a specific immunoassay", HYBRIDOMA Volume 9, Number 2, 1990, pp177-187 Abstract, p185	1-22
P, A	AU A 93559/98 (ELLIS et al) 12 April 1999	1-22

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 1999	17. 12. 99

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**ROSS OSBORNE** Telephone No.: (02) 6283 2404

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00995

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member		
AU	93559/99	WO	99/15667	01.04.99

END OF ANNEX